resolution. Finally, combination of gCW-STED with fluorescent correlation spectroscopy (FCS) is discussed: gCW-STED offers the unique property of tuning the effective detection volume by sorting photons in time.

[1] Vicidomini G, Moneron G, Han KY, Westphal V, Ta H, et al. (2011) Sharper low-power STED nanoscopy by time gating. Nat Methods 8: 571-573.

3429-Pos Board B584

A Hypsochromic Shift Observed in Far-Red Cyanine Dyes Leads to Artifacts in Quantitative Super-Resolution Imaging

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Far-red cyanine dyes such as Alexa 647, Cy5, and Atto 647 are favored for multi-color super-resolution microscopy due to their long wavelength fluorescence and their favorable reversible photo-switching characteristics. Conjugation of these dyes to proteins can result in unwanted fluorescence effects such as shifts in absorption bands and fluorescence quenching (1). We additionally observe a ~200 nm hypsochromic shift in the emission from a small fraction of antibody fragments conjugated to far-red cyanine dyes when probes are excited at 532 nm or 560 nm. This state is nearly undetectable in bulk fluorescent measurements, but is easily detected in live cell super-resolution experiments where we observe a long lived fluorescent state that is resistant to bleaching. This population is present in pre-conjugated secondary antibodies purchased from commercial sources, as well as in antibody fragments conjugated under dilute conditions. Unfortunately, this blue-shifted emission closely resembles that of the red-shifted state of the popular mEOS2/mEOS3.2 photo-switchable probes, making it difficult to distinguish these labels in an imaging experiment. We will describe our efforts to devise practical methods to reduce possible artifacts associated with this state in multi-color super-resolution fluorescence localization measurements.

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3430-Pos Board B585

Isotropic 3D Super Resolution Imaging with Self-Bending Point Spread Function

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Recent years have seen the emergence of super-resolution imaging techniques. Among these methods, single-molecule based STORM or (F)PALM depends on precise localizations of individual emitters in all dimensions. Various three-dimensional (3D) imaging strategies have been reported. Here we report a novel 3D super-resolution imaging method with isotropic 3D resolution over long imaging depth. We achieved this imaging method by designing and experimentally implementing a self-bending point spread function (SB-PSF) based on the nondiffracting Airy beam. The unique property of the beam allows the new PSF to exhibit little diffraction over long distance, as well as experiencing constant self-bending (transverse acceleration) during propagation. In this sense, axial information of an emitter is revealed by its lateral bending to a high accuracy. Experimentally, SB-PSF expands only 2.4 times over a depth of field of 3μm, compared to >15 times of a conventional PSF. Isotropic FWHM resolutions of 20-30 nm are achieved in all three dimensions over the depth of field with an average of 2000 photons detected per fluorophore and substantially better resolutions are possible with brighter fluorophores. The results are in well agreement with numerical simulations derived from a nondiffracting solution to the wave equation. We demonstrated 3D super-resolution with the SB-PSF on a variety of cellular structures. This novel PSF should also greatly benefit other imaging modes such as confocal imaging and single-partical tracking, etc.

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Automated Algorithm for Quantitative Analysis of Fluorescence Nanoscopy Images

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Fluorescence nanoscopy, both in the far-field (STED) and near-field version (NSOM) allows imaging of biological samples with exquisite resolution and high signal-to-noise ratio, representing a powerful tool to address biological questions at the nanometer scale. Yet, despite the highly improved resolution and quality in comparison with conventional confocal microscopy, Poisson noise, background noise and cell autofluorescence are limiting factors for the automated quantification of molecular spatial organization as well as for the recognition of distinct features such as clusters and aggregates. For these reasons, the analysis of nanoscopy images, if at all, is often performed in a semi-automatic fashion, requiring the manual identification of image features and the supply of user-defined parameters. As such, these approaches are time-consuming and might suffer from non-objective evaluation, which are critical parameters in the analysis of samples with high emitters density.

Here we propose a method that allows fully automated reconstruction of fluorescence nanoscopy images and quantitative analysis of protein spatial organization. Our approach is based on the combination of a maximum likelihood algorithm and an expectation-maximization step, by means of which the image is de-noised and decomposed into point-spread functions. The further application of a local maxima identifying routine and Voronoi tessellation, allow us to extract the maximum information from the fluorescence images without impacting on the optical resolution. The performance of the method has been tested on simulated images at varying emitter density and signal-to-noise ratio, showing its applicability to standard STED and NSOM images. Furthermore, we have successfully applied this algorithm to quantitatively analyze fluorescence nanoscopy images of high densely packed membrane receptors on mammalian cells. Our results show faithful retrieval of receptor positions, stoichiometry and their lateral distribution of receptors on the cell membrane.

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Calibration on the Spot of EMCCD Cameras for Super Resolution Microscopy

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In single-molecule biophysics and super-resolution microscopy, fluorescent probes are routinely localized with nanometer precision in images taken, e.g., with an EMCCD camera. In such images, an isolated probe images as a diffraction-limited spot of light which was formed by a finite number of photons. The probe's coordinates are estimated from the recorded camera intensities in the spot, and the error on this estimate, the localization error, is given by a mathematical formula that depends on the number of photons in the spot. Translation of measured intensities to photon numbers requires a calibration of the camera for the specific setting with which it is used. Here we show how this can be done post festum from just a recorded image. We demonstrate this (i) theoretically, mathematically, (ii) by analyzing images recorded with an EMCCD camera, and (iii) by analyzing simulated EMCCD images for which we know the true values of parameters. In summary, our method of calibration-on-the-spot allows calibration of a camera with unknown settings from old images on file, with no other info needed. Consequently, calibration-on-the-spot also makes future camera calibrations before and after measurements unnecessary, because the calibration is encoded in recorded images during the measurement itself, and can at any later time be decoded with calibration-on-the-spot.

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Simulation and Classification of Multi-State Diffusion on Arbitrary, Non-Euclidean Surfaces

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Signaling and trafficking of membrane proteins in response to binding of extracelluar ligand proceeds through various steps that may include free diffusion, confined diffusion, immobilization, internalization, and intracellular trafficking. The rate at which a protein proceeds through such a sequence may reflect the efficacy of signaling. Each of these steps manifest as distinct types of motion that can be used to identify the state from single-particle tracking data. In order to develop methods for classification of trajectories into distinct states, we have developed a method to simulate the trajectory of a particle as it undergoes various types of motion while confined to a realistic 3D cell membrane geometry.

By applying traditional cubic spline surface fitting algorithms to 3D point cloud data, we have constructed realistic surfaces upon which to simulate multi-state diffusion. We simulated single-particle diffusion on these surfaces via a random walk with a step size determined by the diffusion constant of the state in question. At each step, the random walker is allowed to move a distance equal to the step size in an arc along the fitted surface in the direction of motion. For efficiency, we precomputed the simulated state sequence using the Gillespie algorithm, which has been shown to accurately reproduce, in an ensemble of state sequences, the rates at which the particle changes state.

We investigate the performance of hidden Markov model approaches for classification of states and rate constant estimation from observed trajectories. We also investigate the effect of membrane geometry on state classification when information about axial position is lost, as is typically the case when performing 2D single particle tracking.